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Specific and sensitive PCR-based detection of *Septoria musiva*, *S. populicola* and *S. populi*, the causes of leaf spot and stem canker on poplars

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The development of a PCR assay for the detection of the poplar pathogenic fungi *Septoria musiva* (teleomorph *Mycosphaerella populorum*), *S. populicola* (*M. populicola*) and *S. populi* (*M. populi*) is described. Three pairs of species-specific PCR primers were designed using interspecific polymorphisms in the internal transcribed spacer (ITS) of nuclear ribosomal RNA gene (rDNA) repeats. The specificity of the three primer pairs was successfully tested on a collection of 40 *S. musiva*, 39 *S. populicola* and six *S. populi* isolates. Using stringent PCR conditions, no cross-reaction was observed with any of the isolates tested. The specificity of the PCR assay was further confirmed with DNA extracted from 12 additional *Septoria* species and 17 other fungal species obtained from stems or leaves of poplars. Specific amplification of the fragments for *S. musiva* and *S. populicola* was sensitive relatively to the technique used, detecting as low as 1 pg template DNA, and 10 pg of DNA of the target species in a background of 1 ng of DNA of the other species. Moreover, using DNA purified directly from disrupted conidia, it was possible to detect with a probability of 90 %, using one unique PCR assay, the DNA equivalent of 166 conidia per µl of *S. musiva* and 156 conidia per µl of *S. populicola*. The procedures developed in this work can thus be applied for rapid and accurate detection and identification of *Septoria* species from poplars.

INTRODUCTION

Epidemic leaf spot and stem canker caused by the coelomycete *Septoria musiva* (teleomorph *Mycosphaerella populorum*) are among the most important diseases of hybrid poplars in eastern North America (Ostry 1987, Krupinsky 1989, Royle & Ostry 1995). On highly susceptible clones, *S. musiva* causes necrotic lesions on the leaves which lead to premature defoliation, and cankers on the stem and branches which can reduce growth, predispose the tree to colonisation by secondary organisms, and cause stem breakage (Lo *et al.* 1995). The related *S. populicola* (teleomorph *M. populicola*), which causes endemic leaf spot on native poplars *Populus balsamifera* and *P. trichocarpa* and on their hybrids, may also cause cankers particularly on *P. balsamifera* (Zalasky 1978), but this canker disease may be exceptional or atypical (Newcombe *et al.* 2001).

Knowledge of the host range and the geographic distribution of *S. musiva* and *S. populicola* is essential for evaluating risk of damage and the monitoring of these plant pathogens in hybrid poplar plantations. In eastern North America, the distribution of *S. musiva* is sympatric with native *P. deltoides* on which damage is normally limited to leaf spots (Farr 1991). Endemic leaf spots on this local species are thought to be the initial source of inoculum for new hybrid poplar plantings (Newcombe *et al.* 2001). Leaf spots on willow may also provide an additional source of inoculum as we have recently isolated *S. musiva* from symptomatic leaves of *Salix lucida* spp. *lucida* (Feau & Bernier 2004). Distribution of *S. populicola* is sympatric with *P. balsamifera* and *P. trichocarpa* (Zalasky 1978, Newcombe *et al.* 2001), whereas *S. musiva* is uncommon but suspected on these hosts (Newcombe *et al.* 2001). Furthermore, *P. balsamifera* is distributed throughout northeastern North America, whereas *P. deltoides* is most prevalent in the central and southeastern parts of the continent. The occurrence of *S. musiva* on *P. balsamifera* could facilitate further extension of the leaf spot and canker diseases in northeastern North America (Newcombe *et al.* 2001). Although *S. musiva* was thought to be present throughout North America, there are only unconfirmed reports in the western USA (Ostry 1994,

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Newcombe *et al*. 1995, Royle & Ostry 1995). In this region, it was found that only *S. populicola* was responsible for causing epidemic leaf disease on *P. trichocarpa* and hybrid poplars (Newcombe *et al*. 1995, Royle & Ostry 1995). The indigenous host, *P. trichocarpa*, and its hybrids are susceptible to *S. musiva* (Newcombe *et al*. 1995, Newcombe & Bradshaw 1996) and poplar breeders in the Pacific Northwest are concerned about the impact that its introduction into the region would have on their programs. In order to clarify the geographic distribution and confirm the host ranges of *S. musiva* and *S. populicola*, an accurate method of identification of the two species is required. *Septoria musiva* and *S. populicola* may cause very similar disease symptoms on leaves and have been differentiated for many years based on morphological traits. However, morphometric and morphologic criteria such as conidial length and septation may not be reliable enough for routine diagnosis because of overlap between *S. musiva* (28–54 × 3.5–4 μm; 1–4 septate) and *S. populicola* (45–80 × 3–4 μm; 2–5 septate) (Sivanesan 1990b, Royle & Ostry 1995). In addition, distinguishing the two species on the basis of characters of pseudothecia that are found in fallen overwintered leaves remains impractical because of difficulty in finding the teleomorph in nature (Sinclair, Lyon & Johnson 1987). Therefore, diagnostic methods other than morphological criteria are desirable for the early and reliable detection and identification of *S. musiva* and *S. populicola*.

A third species, *S. populi* (teleomorph *Mycosphaerella populi*), responsible for leaf spot on poplar in Europe, central Asia and South America (Browne 1968, Quraishi & Jamal 1970, Sivanesan 1990a), was included in this study. Occurrence of this species in North America remains uncertain. Although Farr *et al*. (1989) mentioned this species as occurring in Alaska and temperate North America, the accounts of Browne (1968), Quraishi & Jamal (1970) and Sivanesan (1990a) do not include North America in the range of this fungus. Like *S. musiva* and *S. populicola*, *S. populi* causes the formation of small, dark leaf spots which are more pale at the center. Though mostly one septate, the size of conidia of *S. populi* (30–40 × 3–4 μm) is within the range of dimensions of conidia of *S. musiva* (Sivanesan 1990b). Potential morphological confusions and contradictions about the geographic range of *S. populi*, and absence of knowledge about the consequences of an accidental introduction of this species into North America justify the need for a more reliable method to identify this pathogen.

Molecular methods are now commonly used for fungal species identification, especially for large genera that include species which exhibit overlapping morphological characteristics (Grote *et al*. 2002, Henson & French 1993, Martin, Delano & Lévesque 2000). PCR-based techniques provide the opportunity to selectively amplify DNA sequences of an organism at a high level of sensitivity. The ITS of the nuclear ribosomal RNA gene (rDNA) repeats is located on each side of the highly conserved 5.8S region. The ITS region evolves rapidly, shows a high level of polymorphism, and also occurs in multiples copies per haploid genome (Bruns, White & Taylor 1991, Bruns & Szaro 1992). These features make the ITS region useful for studying variation among closely related species (White *et al*. 1990, Gardes & Bruns 1993). The ITS region has been used extensively for the identification of fungal pathogens of forest trees (Hamelin *et al*. 2000, Germain *et al*. 2002), fruit trees (Ioos & Frey 2000), and annual crops (Freeman *et al*. 2002, Reid *et al*. 2002).

The objective of this work was to study the genetic variability within the ITS region of *S. musiva*, *S. populicola*, and *S. populi* in order to design species-specific PCR primer pairs for each of the three species and to establish a qualitative PCR assay with high specificity and sensitivity for their detection. The specificity and the absence of cross-reactivity were tested on a wide range of S*eptoria* species and other fungi occurring on poplar. These tests were independently performed at Université Laval and at University of Wisconsin-Madison laboratories using two different DNA extraction and PCR protocols. The levels of sensitivity for the *S. musiva* and *S. populicola* specific primer pairs were determined with the Université Laval laboratory extraction and PCR protocols and finally, PCR protocols were tested for the capacity to detect *S. musiva* and *S. populicola* directly from conidia and DNA purified from conidia.

MATERIALS AND METHODS

Isolates and cultural conditions

40 isolates of *Septoria musiva*, 39 of *S. populicola*, six of *S. populi*, and 13 representing 12 other *Septoria* species were obtained from herbaceous and woody perennial plants (Table 1). The *S. musiva* and *S. populicola* isolates were sampled among their host and geographical ranges as defined previously in the literature (Zalasky 1978, Farr 1991, Royle & Ostry 1995, Newcombe *et al*. 2001). Isolates from leaf spots were isolated from conidia obtained by crushing pycnidia or from cirri produced after incubation of leaves in a moist chamber. Those collected from cankers were obtained by plating pieces of bark from cankered poplar tissues. In addition, isolates of at least 17 other fungal species were obtained from poplar tissues and grown on potato dextrose agar (PDA) (Table 1). These other fungi were obtained during previous attempts to culture *S. musiva* from cankers on poplars as described in Stanosz & Stanosz (2002) and include genera or species which occur on bark, stem and leaves. *Septoria* isolates collected at the Université Laval were grown initially on PDA and those from the University of Wisconsin-Madison on V8 juice agar. Characterization of isolates obtained by the authors included observation of symptoms on the host and microscopic observation of conidia. *Septoria* species were identified according to

Table 1. Fungal isolates used to design and test PCR primers specific for *Septoria musiva*, *S. populicola* and *S. populi*

Species	Lab[a]	Isolate no.	Host species or *Populus* hybrid[b]	Geographic origin and (year of isolation)	Amplification[c]		
					Smus[d]	Spop	Spn
Septoria musiva	UL/UW	m01.01d[e]	*Populus deltoides*	Leclerville, Québec, CAN (2001)	+	−	−
		m04.01d[e]	*P. deltoides*	Saint-Ours, Québec (2001)	+	−	−
		m01.02j	*P.* × *jackii*	Sainte-Foy, Québec (2002)	+	−	−
		01-73B[e]	*Populus* hybrid – canker[g]	Douglas Co., Minnesota, USA–(2001)	+	−	−
	UL	SO66[e]	750316 (M × J)	Saint-Ours, Québec (2001)	+	−	−
		sal03.01[f]	*Salix lucida* spp. *lucida*	Leclerville, Québec (2001)	+	−	−
		m02.02d	*P. deltoides*	Sainte-Foy, Québec (2002)	+	−	−
		97.04	915508 (E × M) – canker	Québec (1997)	+	−	−
		02-169A[e]	NM6 (N × M)	Calumet Co., Wisconsin, USA (2002)	+	−	−
		02-131A[e]	MWH15 (D × M)	Dane Co., Wisconsin (2002)	+	−	−
		ab11.02[e]	*P.* × *jackii*	Baby, Québec (2002)	+	−	−
		ab09.02	*P.* × *jackii*	Baby, Québec (2002)	+	−	−
		ab14.02	*P.* × *jackii*	Baby, Québec (2002)	+	−	−
		91.04	*Populus* hybrid – canker	Saint-Hugues, Québec (1991)	+	−	−
		91.15	750462 [M × (D × T)] – canker	Lotbinière, Québec (1991)	+	−	−
		93.03	3529 (D × M) – canker	Lotbinière, Québec (1993)	+	−	−
		93.11	4351 (B × M)	Saint-Ours, Québec (1993)	+	−	−
		98.25	*Populus* hybrid – canker	Saint-Ours, Québec (1998)	+	−	−
		nd0102	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		nd0202	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		nd0302	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		nd0402	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		nd0502	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		nd0602	*P. deltoides*	Notre-Dame du Nord, Québec (2002)	+	−	−
		ont0102	NM6 (N × M)	Cornwall, Ontario, CAN (2002)	+	−	−
		ont0102d	*P. deltoides*	Cornwall, Ontario (2002)	+	−	−
		ont0702d	*P. deltoides*	Cornwall, Ontario (2002)	+	−	−
		ont0902d	*P. deltoides*	Cornwall, Ontario (2002)	+	−	−
	UW	92-49[f]	*P.* hybrid	Calumet Co., Wisconsin (1992)	+	−	−
		00-60[e]	*P.* sp.	Washington Co., Wisconsin (2000)	+	−	−
		01-01[e]	(T × D) × M – canker	Livingston Co., Kentucky, USA (2001)	+	−	−
		01-02[e]	(T × D) × M – canker	Livingston, Co., Kentucky (2001)	+	−	−
		01-06[e]	*Populus* hybrid – canker	Pope Co., Minnesota (2001)	+	−	−
		02-106	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-107	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-108	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-109	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-110	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-111	NC11382 (N × B) – canker	Dane Co., Wisconsin (2002)	+	−	−
		02-112	NC11382 (N × B) – canker	Dane Co., Wisconsin (2002)	+	−	−
S. populicola	UL/UW	p07.01b[e]	*P. balsamifera*	Leclerville, Québec (2001)	−	+	−
		p04.01t[e]	*P. tremuloides*	Sainte-Françoise, Québec (2001)	−	+	−
		p02.02b[e]	*P. balsamifera*	Matane, Québec (2002)	−	+	−
		02-79A[e]	*P. trichocarpa*	Clatsop Co., Oregon, USA (2002)	−	+	−
		02-86A[e]	*P. trichocarpa*	Thurston Co., Washington, USA (2002)	−	+	−
		02-80A	*P. trichocarpa*	Clatsop Co., Oregon (2002)	−	+	−
	UL	p01.01b[e]	*P. balsamifera*	Leclerville, Québec (2001)	−	+	−
		p02.01b[e]	*P. balsamifera*	Leclerville, Québec (2001)	−	+	−
		02-69A	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		ul01.01[e]	*P. balsamifera*	Sainte-Foy, Québec (2001)	−	+	−
		02-74C	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		02-78C	*P. trichocarpa*	Clatsop Co., Oregon (2002)	−	+	−
		p01.03h	*Populus* hybrid	Grands-Piles, Québec (2003)	−	+	−
		p06.01b	*P. balsamifera*	Leclerville, Québec (2001)	−	+	−
		abp02.02	3375 (B × M)	Baby, Québec (2002)	−	+	−
		abp03.02	3375 (B × M)	Baby, Québec (2002)	−	+	−
		abp05.02	*Populus* hybrid	Baby, Québec (2002)	−	+	−
	UW	02-70A	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		02-72A	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		02-74A	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		02-75A	*P. trichocarpa*	Marion Co., Oregon (2002)	−	+	−
		02-76A	*P. trichocarpa*	Clark Co., Washington (2002)	−	+	−
		02-81A	*P. trichocarpa*	Clatsop Co., Oregon (2002)	−	+	−
		02-82A	*P. trichocarpa*	Clatsop Co., Oregon (2002)	−	+	−

Table 1. (*Cont.*)

Species	Lab[a]	Isolate no.	Host species or *Populus* hybrid[b]	Geographic origin and (year of isolation)	Amplification[c]		
					Smus[d]	Spop	Spn
		02-83A	*P. trichocarpa*	Cowlitz Co., Washington (2002)	–	+	–
		02-84A	*P. trichocarpa*	Cowlitz Co., Washington (2002)	–	+	–
		02-87A	*P. trichocarpa*	Thurston Co., Washington (2002)	–	+	–
		02-88A	*P. trichocarpa*	Thurston Co., Washington (2002)	–	+	–
		02-89A	*P. trichocarpa*	Thurston Co., Washington (2002)	–	+	–
		02-90A	*P. trichocarpa*	Pierce Co., Washington (2002)	–	+	–
		02-91A	*P. trichocarpa*	Pierce Co., Washington (2002)	–	+	–
		02-93	*P. trichocarpa*	Marion Co., Oregon (2002)	–	+	–
		02-94	*P. trichocarpa*	Marion Co., Oregon (2002)	–	+	–
		02-96	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
		02-97	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
		02-98	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
		02-99	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
		02-100	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
		02-101	*P. trichocarpa*	Clatsop Co., Oregon (2002)	–	+	–
S. populi	UL/UW	pn01.02[e]	*P. nigra*	Southeastern France (2002)	–	–	+
		pn02.02[e]	*P. nigra*	Southeastern France (2002)	–	–	+
		pn03.02[e]	*P. nigra*	Southeastern France (2002)	–	–	+
		pn05.02[e]	*P. nigra*	Southeastern France (2002)	–	–	+
	UL	pn04.02[e]	*P. nigra*	Southeastern France (2002)	–	–	+
		pn01.03[e]	*P. nigra*	Croatia (2003)	–	–	+
S. alnifolia	UL	aln01.02	*Alnus incana* ssp. *rugosa*	Sainte-Foy, Québec (2002)	–	–	–
S. sambuccina		sam02.01	*Sambuccus pubescens*	Sainte-Françoise, Québec (2001)	–	–	–
S. ribis		rib01.01	*Ribes* sp.	Camp Mercier, Québec (2001)	–	–	–
S. betulae		98-37B	*Betula* sp.	Dane Co., Wisconsin (2001)	–	–	–
		bpop01.01	*Betula populifolia*	Sainte-Foy, Québec (2001)	–	–	–
S. lycopersicii		lyc01.01	*Lycopersicon esculentum*	Wisconsin (2001)	–	–	–
S. albopunctata		alb02.02	*Vaccinium ashei* cv. 'Brightwell'	Georgia, USA (2002)[h]	–	–	–
S. glycines		sgly01.02	*Glycines max*	Ontario, CAN (2001)[i]	–	–	–
S. helianthi		02-252 A	*Helianthus* sp.	Dane Co., Wisconsin (2002)	–	–	–
S. canadensis		can03.02	*Cornus canadensis*	Stoneham, Québec (2002)	–	–	–
S. cornicola		cor01.02	*C. stolonifera*	Thetford-Mines, Québec (2002)	–	–	–
S. floridae		fl01.02	*C. florida*	Michigan, USA (2002)	–	–	–
S. ostryae		ost01.01	*Corylus* sp.	Stoneham, Québec (2001)	–	–	–
Acremonium sp.	UW	02-39	*Populus* hybrid – canker	Columbia Co., Wisconsin (2002)	–	0	0
Alternaria sp.		02-22	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-23	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Cladosporium sp.		02-05	*P. trichocarpa*	Washington (2002)	–	–	–
		02-06	*P. trichocarpa*	Washington (2002)	–	–	–
		02-07	*P. trichocarpa*	Washington (2002)	–	–	–
		02-08	*P. trichocarpa*	Washington (2002)	–	–	–
		02-21	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Colletotrichum sp.		02-24	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-30	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-33	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-35	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Cryptodiaporthe sp.		02-54	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-56	*Populus* hybrid – stem	Columbia Co., Wisconsin (2002)	–	–	–
Cytospora chrysosperma		92-57	*P. tremuloides* – canker	Colorado, USA (2002)[j]	–	–	–
Epicoccum sp.		02-18	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-19	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-20	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Fusarium acuminatum		02-103	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	–	–	–
Fusarium equiseti		02-36	*Populus* hybrid – canker	Columbia Co., Wisconsin (2002)	–	0	0
Fusarium solani		02-41	*Populus* hybrid – canker	Columbia Co., Wisconsin (2002)	–	–	–
Phomopsis sp.		02-26	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-27	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-31	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Sclerotinia sp.		02-25	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Sphaeropsis sp.		99-2A	*P. deltoides* – stem	Columbia Co., Wisconsin (2002)	–	–	–
Unknown sp. 1		02-38	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
Unknown sp. 2		02-55	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–

Table 1. (*Cont.*)

Species	Lab[a]	Isolate no.	Host species or *Populus* hybrid[b]	Geographic origin and (year of isolation)	Amplification[c] Smus[d]	Spop	Spn
Unknown pycnidial sp.		02-28	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-29	NM6 (N × M) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-32	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-34	DN34 (D × N) – stem	Columbia Co., Wisconsin (2002)	–	–	–
		02-37	*Populus* hybrid – canker	Columbia Co., Wisconsin (2002)	–	–	–
Verticillium sp.		02-104	NC11505 (M × T) – canker	Dane Co., Wisconsin (2002)	–	–	–

[a] Isolates tested at Université Laval (UL) or/and University of Wisconsin-Madison (UW).
[b] Host species or *Populus* hybrid. When known, parentage is indicated in parentheses as follows: *P. berolinensis* (B); *P. deltoides* (D); *P.* x*euramericana* (E); *P.* ×*jackii* (J); *P. maximowiczii* (M); *P. nigra* (N); *P. trichocarpa* (T).
[c] +, present; −, absent; 0, isolate not been tested with the respective primer pair.
[d] Primer pairs specific to *S. musiva* (Smus), *S. populicola* (Spop) and *S. populi* (Spn).
[e] Sequenced in this study and used in sequence analysis.
[f] Sequences obtained from the International Nucleotide Sequence Database Collaboration database.
[g] Unless otherwise specified, isolates were obtained from leaf spots.
[h] Provided by Harald Sherm (Department of Plant Pathology, University of Georgia).
[i] Provided by Terry Anderson (Agriculture Canada, Greenhouse and Processing Crops Research, Harrow, Ontario).
[j] Provided by William Jacobi (Department of Bioagricultural Sciences and Pest Management, Colorado State University).

Constantinescu (1984), Farr (1991), Ellis & Ellis (1985) and to the *CMI Descriptions* (Holliday & Punithalingam 1970, Punithalingam & Holliday 1972, Sivanesan 1990a, b). The conidial sizes of all the *S. musiva*, *S. populicola* and *S. populi* isolates used in the study were within their specific range of dimensions.

Isolates collected at the University of Wisconsin-Madison were stored at 5 °C on PDA in slant tubes with screw caps. Mycelium plugs in caps were covered with sterile mineral oil. Those sampled at Université Laval were kept at −80 ° in potato dextrose broth containing 15% glycerol.

rDNA sequence analysis and primer design

The ITS region of 11 isolates of *Septoria musiva*, eight of *S. populicola*, and six isolates of *S. populi* representative of different symptoms, host and geographical regions (Table 1) was sequenced and compared by multiple alignment. Additionally, the ITS sequences of the *S. musiva* isolates 92–49 (accession no. AF243392), sal03.01 (AY555277), AF243391 and AF216533 and those of five *S. populicola* isolates (AY152583 to AY152587) were obtained from the International Nucleotide Sequence Database Collaboration database (downloaded from the NCBI databases website: http://www.ncbi.nlm.nih.gov/) and added to the multiple alignment. Cultural conditions, DNA extraction procedures, and PCR conditions were developed at University Laval as described below. Mycelium from *Septoria* was grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid V8-juice medium with 0.2% $CaCO_3$. The flasks were seeded with two mycelium plugs, placed on a rotary shaker at 110 rev min^{-1} and incubated for 1 wk at 22 ° in darkness. The liquid cultures were centrifuged at 4700 ***g*** for 10 min; the supernatant was removed and the pellets were washed with sterile distilled water, lyophilised and ground in liquid nitrogen. Total genomic DNA was extracted with the hexadecyltrimethylammonium bromide (CTAB) method according to Zolan & Pukkila (1986). Total DNA was diluted 30-fold in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and used as template for PCR amplification.

The full-length ITS (ITS1, ITS2 and the 5.8S rDNA) was amplified from the total genomic DNA with the universal primers ITS5 and ITS4 (White *et al.* 1990). Amplifications were performed in 20 µl reactions containing 1× *Taq* polymerase buffer (20 mM Tris-HCl pH 8.5 mM KCl), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Roche Diagnostics, Penzberg), 0.2 mM of each dNTP, 0.2 µM of each primer, and 2 µl of template DNA. PCR amplifications were performed in a MJResearch thermal cycler model PTC100 (Incline Village, NV) with an initial denaturation (94 °, 2 min) followed by 30 cycles of denaturation (94 °, 30 s), annealing (55 °, 30 s), extension (72 °, 1 min) and a final extension (72 °, 10 min). The PCR products were separated by electrophoresis on 1% agarose gel in 0.5× TAE buffer. Gels were stained with ethidum bromide and photographed under UV light. The amplified fragments were cleaned with a QIA Quick purification kit (Qiagen, Mississauga, ON). DNA concentration was measured by a quantitative fluorimetric assay with the fluorochrome Hoechst 33258 (Labarca & Paigen 1980) using a TKO 100 DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Both strands of the purified amplified DNA fragments were sequenced in an ABI 377 automated DNA sequencer by the dideoxynucleotide chain termination procedure using the appropriate amplification primers and a Sequenase GC-rich kit (Applied Biosystems, Foster City, CA). Multiple sequence alignment with hierarchical clustering was performed using CLUSTAL X (Thompson *et al.* 1997) and edited using BioEdit software (Hall 1999). Specific primer pairs for *S. musiva*, *S. populicola*

and *S. populi* were designed using Primer3 software (Rozen & Skaletsky 2000) and synthesized by Operon Technologies (Alameda, CA).

Sequence data of isolates sequenced in this study were deposited in GenBank under accession nos. AY549464–AY549478.

Specificity of PCR amplification

Primer pair specificity was evaluated by searching the International Nucleotide Sequence Database Collaboration database using the BLAST program (short, nearly exact matches) (website: http://www.ncbi.nlm.nih.gov/blast/) and submitting each primer string separately.

In order to test and routinely use the species-specific primers, two PCR amplification protocols were independently developed at Université Laval and at University of Wisconsin-Madison. Cultural and PCR conditions chosen to amplify species specific fragments from total genomic DNA of *Septoria* species at Université Laval were as described above with the following modifications. Parameters for the specific amplification of DNA for *S. populicola* and *S. populi* were optimized by increasing the concentration of primers to 0.4 μM. Annealing temperatures were 64 ° for the primer pairs specific to *S. musiva* and to *S. populicola*, and 61 ° for the primer pair specific to *S. populi*. Cultural conditions, DNA extraction procedures, and PCR conditions were modified at University of Wisconsin-Madison as described below. Mycelia of *S. musiva* and *S. populi* were grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid V8-juice medium with 0.2 % $CaCO_3$ on a rotary shaker at 110 rev min^{-1} at ambient laboratory conditions. Mycelia of *S. populicola* and other fungi obtained from poplar stems and leaves were grown in 50 ml potato dextrose broth (PDB) in 125 ml Erlenmeyer flasks incubated with shaking as described above. Mycelia were collected after 1–2 wk by centrifugation at 8000 ***g*** for 5 min. The supernatant was removed and pellets were washed with 10 mM Tris-1 mM EDTA. Total genomic DNA was extracted with the method described by Smith & Stanosz (1995), diluted 50–100-fold in milliQ water, and used as template for PCR amplification. Amplification was performed in 25 μl reactions containing 1× buffer, 1 mM $MgCl_2$, 1 U *Taq* DNA polymerase, 0.15 mM of each dNTP, 1.0 μM of each appropriate primer, and 2 μl of template DNA. PCR amplifications were performed in a Perkin-Elmer Thermal Cycler 480 (Norwalk, CT) with an initial denaturation (95 °, 5 min) followed by 30 cycles of denaturation (95 °, 1 min), annealing (66 °, 1 min), extension (72 °, 1 min) and a final extension (72 °, 5 min). The annealing temperatures were 59 ° for the *S. populicola* primer pair and 55 ° for the *S. populi* primer pair. The PCR products were separated by electrophoresis on 0.7 % agarose gel in 0.5× TBE buffer. Gels were stained with ethidium bromide and photographed under UV light.

Sensitivity of species-specific PCR amplification

In order to determine the sensitivity of the PCR assay specific to *Septoria musiva* and *S. populicola*, PCR experiments were conduced on serial dilutions of DNA extracted from mycelia and on serial dilutions of conidia or DNA extracted and purified from serial dilution of conidia.

DNA solutions from mycelia were prepared which contained: (1) 10-fold dilutions of DNA (1 ng to 1×10^{-5} ng) from *S. musiva* or *S. populicola* mycelia; or (2) a constant amount of 1 ng of DNA from mycelia of one of the two species mixed with 10-fold dilutions of DNA from mycelia (1 ng to 1×10^{-5} ng) of the target species. The initial DNA concentrations were determined and adjusted following the fluorimetric assay described above (Labarca & Paigen 1980).

Serial dilutions of conidia and DNA extracted and purified from serial dilution of conidia were prepared as described below. Stock conidial suspensions from 10 d cultures of *S. musiva* (isolate 97.04) and *S. populicola* (isolate p02.02b) grown on PDA were prepared and enumerated by direct microscopic counting in a haemocytometer. Conidial suspensions were prepared which contained: (1) serial dilutions of conidia (500, 250, 125, 75, 50, 25 and 5 conidia μl^{-1}) from *S. musiva* or *S. populicola*; or (2) a constant amount of 500 conidia per μl from one of the two species mixed with serial dilutions of conidia (500, 250, 125, 75, 50, 25 and 5 conidia μl^{-1}) from the target species. Three different methods of preparing the conidial suspension samples for PCR assay were compared: (1) 2 μl of molecular-biology-grade-water containing untreated conidia were added directly to the PCR amplification mixture; (2) 2 μl of a suspension containing conidia mechanically disrupted with glass beads were added to the PCR amplification mixture; and (3) total genomic DNA extracted from mechanically disrupted conidia was purified and used for PCR amplification. For Methods 2 and 3, conidial suspensions were prepared in 200 μl of Tris-EDTA-CTAB buffer in a 2 ml microfuge tube with a screw cap. Total genomic DNA from conidia was extracted and purified by a method modified from Lee & Taylor (1990). Disruption of spores was achieved by shaking spore suspensions with ten to twelve 3 mm glass beads. The tubes were then vigorously shaken for 3 min at maximum speed on a Retch MM-300 shaker (Newton, PA), prior to being incubated for 1 hour at 65 °. The supernatant above the beads was then recovered for the species-specific PCR analyses. Microscopic examination of conidial suspensions before and after these treatments showed that they disrupted more than 50 % of the *Septoria* conidia. For purification, DNA extracted from disrupted conidia was then precipitated overnight at −20 ° after the addition of 150 μl of isopropyl alcohol and centrifuged for 30 min at 14 000 ***g***. DNA pellets were dried 30 min before being washed with 500 μl of 95 % cold ethanol and centrifuged for 3 min at 14 000 ***g***. Total purified DNA was

diluted 5-fold in molecular-biology-grade-water. PCR assays used 2 μl of a suspension of disrupted conidia or total DNA solution extracted from conidia. Negative controls, using only the reagents, were included in each preparation.

The probability of detecting DNA extracted and purified from conidial suspensions of *S. musiva* and *S. populicola* was established by performing sixty-eight independent PCR experiments. These were carried out for each of the two conidial treatments coupled with each of the two primer sets Smusf/r or Spopf/r. Conidial treatment corresponded to: (1) serial dilutions of pure conidia (see above) from *S. musiva* or *S. populicola* or; (2) mixture of 500 conidia per μl from one of the two species with serial dilutions of conidia (see above) from the target species. The results of these replicates were used to estimate the probability $p(x)$ of detecting DNA extracted from different conidial concentrations, (x), of a specific species. The logistic regression model with binomial error and identity link function (Agresti 1996) was used to model the probability of detecting a specific concentration of conidia according to the following equation:

$$p(x) = [\exp(\alpha + \beta x]/[1 + \exp(\alpha + \beta x)]$$

where α is the baseline intercept and β the slope of the line representing the change in the probability per unit change in x. The regression parameters α and β were estimated using the maximum likelihood method from the independent PCR observation on each combination of primer pair and conidia treatment (Agresti 1996). The statistical analysis software SAS, Version 8.1 (SAS Institute, Cary, NC) was used for the modeling.

RESULTS

rDNA sequence analysis and primer design

Alignment of the ITS1, 5.8S and ITS2 nucleotide sequences revealed a low level of intraspecific polymorphism within *Septoria musiva*, *S. populicola* and *S. populi*. Indeed, the cladogram generated from the sequence alignment using the Jukes and Cantor genetic distances (Jukes & Cantor 1969) between pairwise isolates generally showed short, poorly supported branches (bootstrap values $<70\%$) within the species clades. The only intraspecific branch that was well-supported was found within the *S. musiva* clade (bootstrap value of 98%) and resulted from a difference of three substitutions and one gap between the two isolates AF243391 and AF216533 and the other 11 isolates. One additional substitution located at position 181 occurred within the *S. musiva* clade and differentiated the two isolates 01-02 and 00-60 from the 11 others; this difference did not result in a well-supported branch for the two isolates (bootstrap value under 70%). In spite of these intraspecific differences, all the *S. musiva* isolates showed more than 99% of sequence identity and Jukes and Cantor distances between isolates varied from 0.000 to 0.007 (Fig. 1).

Similarly, the sequences obtained for the six *S. populi* isolates shared more than 99% identity (Jukes and Cantor distances from 0.000 to 0.006) with only two substitutions located in the ITS1 region (Fig. 1). In the case of *S. populicola*, the 13 sequences analyzed (including the five sequences obtained from the NCBI databases) shared between 97% and 99% identity (Jukes & Cantor distances from 0.000 to 0.007) with six nucleotide substitutions locations out of the entire length of 459 bp.

In contrast, the nodes of each of the three morphological species were well supported with bootstrap values ranging from 94% to 100% indicating three distinct clades for the ITS1, 5.8S and ITS2 regions (Fig. 1). A comparison between the ITS1, 5.8S and ITS2 regions of *S. musiva* and *S. populicola* revealed seven single nucleotide substitutions and one gap located at one nucleotide position, representing 94% to 97% identity between these two *Septoria* species. Four base variations were chosen for designing specific primers for each of the two species. One was located in the ITS1 region (base 45; Fig. 2), another one at the 3′ end of the 5.8S region (base 340; Fig. 2) and the two others on the beginning of the ITS2 region (bases 341–345; Fig. 2). The restricted choice of interspecific polymorphic sites within the ITS1 region between *S. musiva* and *S. populicola* led us to design the forward primer specific to *S. populicola* targeting a region containing an intraspecific mutation. This point mutation (38th position relative to our alignment) is located on the 8th nucleotide before the 3′ end of the primer binding site (Fig. 2). The PCR products generated using each of the two pairs of primers (Smusf/Smusr specific for *S. musiva*; Spopf/Spopr specific for *S. populicola*) were, as expected, 329 bp long. The six isolates of *Septoria populi* showed 36 nucleotide differences with the *S. populicola* isolates and 44 differences with the *S. musiva* isolates. An insertion of five nucleotides located in ITS1 (bases 29–34; Fig. 2) and five nucleotide substitutions clustered in ITS2 (bases 436–441; Fig. 2) were chosen to design specific primers for *S. populi*. Primer pair Spnf/Spnr specifically amplified a 439 bp fragment of the nuclear rDNA from *S. populi*.

Specificity of PCR amplification

The BLAST search with the Smusf/r primer strings submitted separately retrieved as the most similar fungal sequences the four *Mycosphaerella populorum* isolates AF243391, AF243392 (isolate 92–49), AF216533 and AY555277 (isolate sal03.01) (100% identity with the 18 bp Smusf string and 100% identity with the 21 bp Smusr string). Other relevant alignments with fungal sequences was found for the Smusf string with isolates of *Eremothecium gossypii* accession numbers NM_209208 and AE016889 (100% identity on 17 bp) and for the Smusr string with an unidentified epacrid

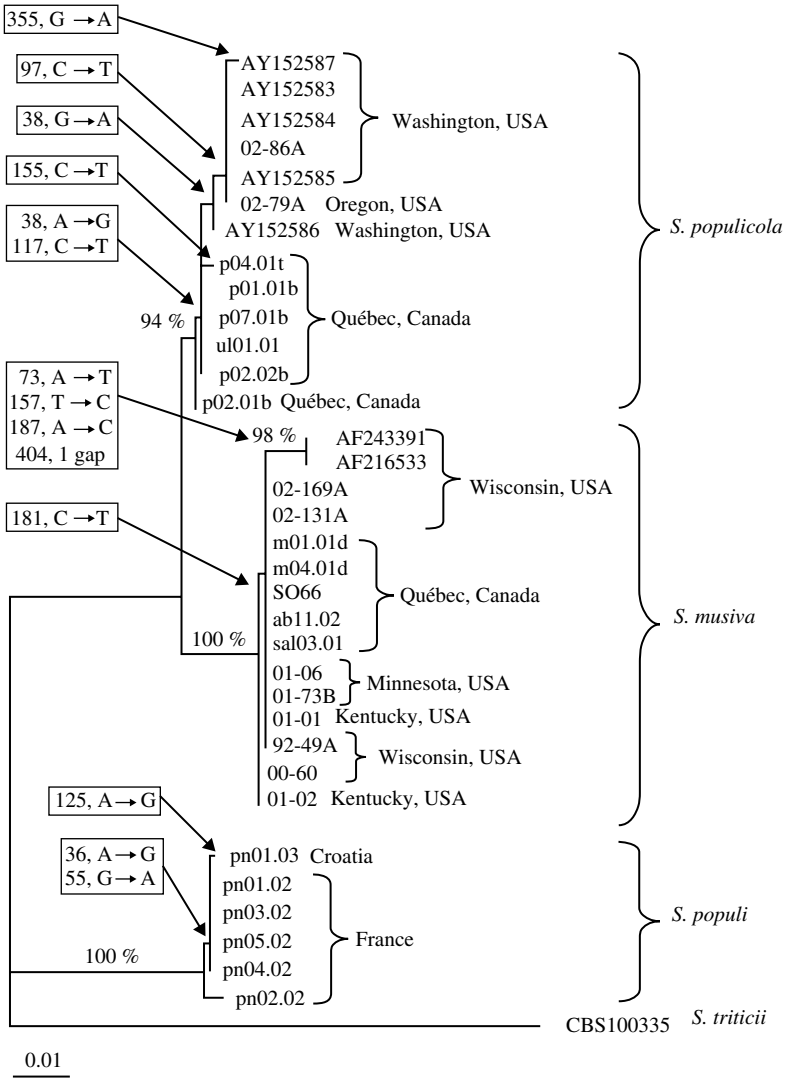

Fig. 1. Neighbour-joining tree obtained using the ITS1, 5.8S and ITS2 nucleotide sequences obtained in this study and those downloaded from the International Nucleotide Sequence Database Collaboration database (indicated with accession numbers). The Juke and Cantor distance model (Jukes & Cantor 1969) was determined as the best fitting model using Modeltest 3.04 (Posada & Crandall 1998) on the 495 bp alignment and was implemented in PAUP* (Swofford 2002) to estimate sequence divergences. Only bootstrap values from 5000 replicates up to 70 % are indicated above branches. Each box with arrow indicates the localization of intraspecific polymorphisms between isolates and contains the substitution position relatively to our alignment (Fig. 2) followed by the type of change. The scale bar indicates horizontal branch length, expressed as the hypothesized number of substitutions per site.

root endophyte (AY279186), and an ericoid endophyte (AF252841) (100 % identity on 19 bp with the two sequences). The BLAST search with the 18 bp string of primer Spopf as query also retrieved as the most similar fungal sequences the isolates of *E. gossypii* accession numbers NM_209208 and AE016889 (100 % identity on 18 bp) and the previous *M. populorum* sequences found with Smusf (100 % identity on 17-bp). On the other hand, query with the 20-bp string of the primer Spopr retrieved the five published sequences of *S. populicola* AY15283 to AY15287 (100 % of identity on the 20-bp), and 216 nuclear rDNA fungal sequences with 100 % of identity on the 20-bp. These 216 sequences represented 204 dothideomycetous fungi including 61 different species (38 *Mycosphaerella* species, nine *Septoria* species and 23 Deuteromycetous species with putative *Mycosphaerella* teleomorphs) and 12 isolates representing six unidentified fungi species. Within these 216 sequences and the 784 others retrieved with a lower match score value, no *M. populorum* sequence was found. Concerning Spnf/r, the specificity of this primer pair was supported by the absence of significant homology with fungal sequences for the Spnr string. The BLAST search with this Spnf 21-bp string as query retrieved the *M. populorum* AF243391, AF243392, AF216533 and AY555277 and the *M. populicola* AY152583 to AY152587 sequences (100 % identity on 16-bp) of Verkley *et al.* (2004). Except for the expected target sequences of *M. populorum* (AF243391, AF243392, AF216533, AY555277), no

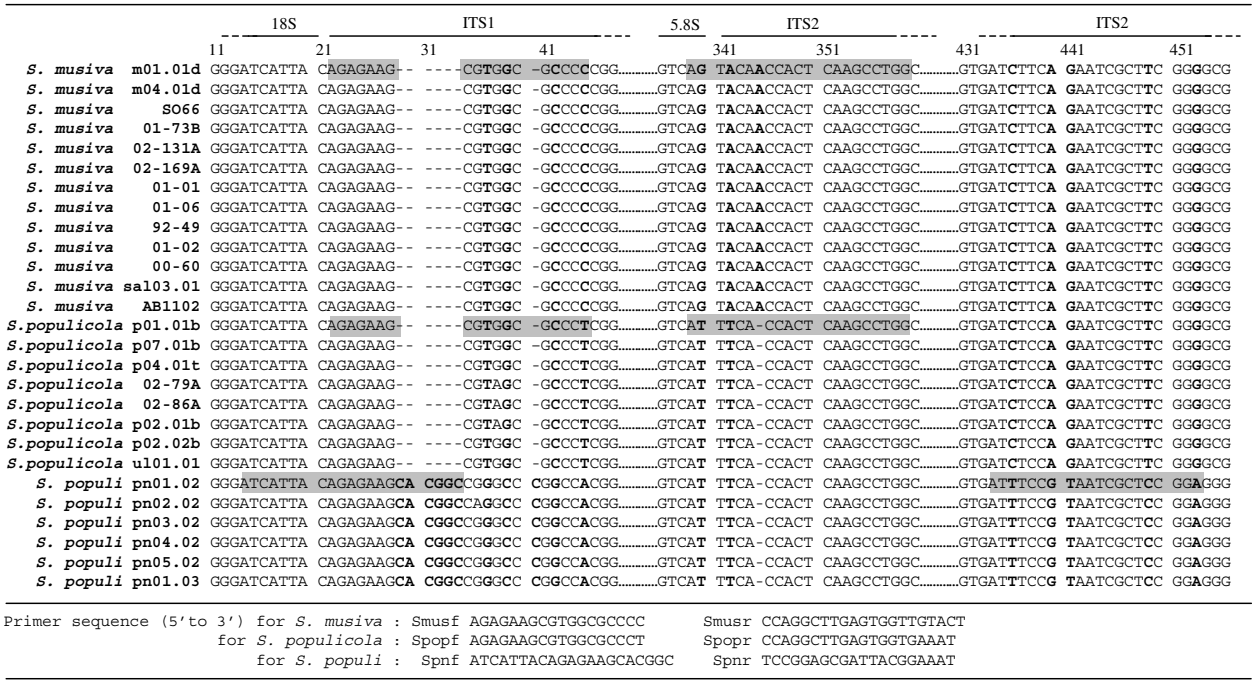

Fig. 2. Sequence alignment of the ITS1 and ITS2 regions for the isolates sequenced in this study showing species-specific base substitutions (in bold). The regions chosen for designing *Septoria musiva*, *S. populicola* and *S. populi* – specific primers are shaded.

other BLAST results were common to both string searches for Smusf/r and Spopf/r, supporting specificity of these primer pairs.

The specificity of the three primer pairs was tested against total genomic DNA extracted using mycelia from isolates of *Septoria musiva*, *S. populicola* and *S. populi*, from a range of *Septoria* species and from other fungal species obtained from leaves or stems of poplar species. Two DNA extraction and PCR amplification protocols using the same primer pairs that were independently tested in our laboratories gave the same results when common isolates were tested (Table 1). When testing isolates of *S. musiva*, *S. populicola* and *S. populi*, no cross-reaction was observed with any isolate, whereas every isolate yielded a single amplification product with only one primer pair. Furthermore, an additional set of 44 *S. musiva* isolates collected over seven North American populations gave the 329 bp expected PCR product with the primer pair specific to *S. musiva* and negative results with the other two primer pairs (data not shown). Although the *S. populicola* 02-86A, 02-79A and p02.01b sequences revealed an intraspecific mutation on the 8th nucleotide before the 3′ end of the primer binding site (Fig. 2), these isolates were amplified successfully in the two laboratories with specific primers Spopf/Spopr, like the four other isolates of *S. populicola* for which ITS sequence data were obtained. Moreover, no amplification was obtained with any of the other fungal species tested (Table 1).

In addition, five DNA samples extracted from field collected poplar leaves (DL22: *P. deltoides*; B1-18: *P. balsamifera*; M77594: *P. maximowiczii*; N42: *P. nigra*; TR666900: *P. trichocarpa*) were tested with the three primer pairs developed in this study. No amplicons were obtained from these DNA samples amplified with the Smusf/r, Spopf/r and Spnf/r primer pairs, though the ITS1 regions were successfully amplified with the ITS1/ITS2 universal primer pair used as a positive control (White *et al*. 1990).

Sensitivity of PCR amplification

The species-specific PCR assays for *Septoria musiva* and *S. populicola* were found to be sensitive, detecting as low as 1 pg template DNA (Fig. 3). Moreover, mixtures of *S. musiva* and *S. populicola* DNA were assayed. In repeated experiments, as little as 10 pg of DNA from one species was detected in a sample (1 ng) of DNA from the second species. For both the *S. musiva and S. populicola* assays, the intensity of the 329 bp fragment decreased gradually with DNA quantities of the target species used in the mixture (Fig. 3).

When untreated or disrupted conidial suspensions were added directly to the PCR mixture, the sensitivity and the accuracy of detection were low. Indeed, by adding directly conidia to the PCR mixture, no positive result was obtained at any of the concentrations tested (500, 250, 125, 75, 50, 25 and 5 conidia μl^{-1}) (data not shown). Consequently, higher conidial concentrations were tested and never fewer than 5000 conidia μl^{-1} were detected. However, at 5000 conidia μl^{-1}, target DNA was not consistently amplified over all the PCR experiments performed. The addition of $\leqslant 5000$ disrupted conidia μl^{-1} directly in the PCR mixture resulted in no detection of *S. musiva* or *S. populicola* DNA (data not shown). When purified DNA from

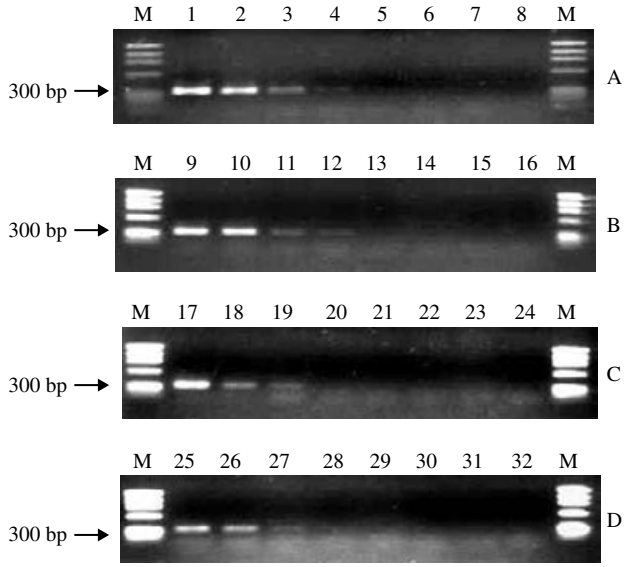

Fig. 3. Sensitivity of PCR protocols for detection of *Septoria musiva* and *S. populicola* genomic DNA in serial dilutions. Amplification of DNA extracted from mycelia of *S. musiva* (isolate S97.04) or *S. populicola* (isolate p02.02b) serially diluted using the pair of primers Smusf/Smusr (A) and Spopf/Spopr (B). PCR reaction mixture contained 1 ng (lane 1), 1×10^{-1} ng (lane 2), 1×10^{-2} ng (lane 3), 1×10^{-3} ng (lane 4), 1×10^{-4} ng (lane 5), 1×10^{-5} ng (lane 6) of *S. musiva* DNA and 1 ng (lane 9), 1×10^{-1} ng (lane 10), 1×10^{-2} ng (lane 11), 1×10^{-3} ng (lane 12), 1×10^{-4} ng (lane 13), 1×10^{-5} ng (lane 14), of *S. populicola* DNA. Amplification of DNA extracted from mycelia of the target species serially diluted in 1 ng of DNA prepared from the other species using the pair of primers Smusf/Smusr (C) and Spopf/Spopr (D). PCR reaction mixture contained 1 ng (lane 17), 1×10^{-1} ng (lane 18), 1×10^{-2} ng (lane 19), 1×10^{-3} ng (lane 20), 1×10^{-4} ng (lane 21), 1×10^{-5} ng (lane 22), of *S. musiva* DNA (isolate 97.04) mixed with 1 ng of *S. populicola* DNA (isolate p02.02b) and 1 ng (lane 25), 1×10^{-1} ng (lane 26), 1×10^{-2} ng (lane 27), 1×10^{-3} ng (lane 28), 1×10^{-4} ng (lane 29), 1×10^{-5} ng (lane 30), of *S. populicola* DNA (isolate p02.02b) mixed with 1 ng of *S. musiva* DNA (isolate 97.04). Negative controls without DNA (lanes 8, 16, 24, 32) and negative DNA controls with 1 ng of *S. musiva* (lanes 15 and 31) or *S. populicola* (lanes 7 and 23) DNA were also included. M: Phi 174/*Hae* III Marker (Promega, Madison, WI).

disrupted conidia of either *S. musiva* or *S. populicola* was used the PCR assays were able to detect DNA from as little as 5 conidia μl^{-1} of the target species and as little as 25 conidia μl^{-1} of the target species in the presence of DNA from 500 conidia μl^{-1} of the competing species (data not shown). From the 68 independent PCR observations made for each primer pair (Smusf/r and Spopf/r) combined with the two conidial treatments (serial dilutions of pure conidia from one species or serial dilution of conidia from one species mixed with 500 conidia per μl^{-1} from the other species), the regression parameters alpha and beta were estimated using the maximum likelihood for each combination of primer pair and conidial treatment (Table 2). In all cases, the logistic regression models fitted the experimental results well. Therefore, these models could be used to describe the detection probability of the primer pairs used for each conidial treatment. Inserting these values into the logistic regression equation gave, for each combination of primer pairs and treatment, the functions illustrated in Fig. 4. From these results, it was determined that the practical operating range (yielding a detection probability higher than 90%) was 166 conidia μl^{-1} for the *S. musiva* assay and 156 conidia μl^{-1} for the *S. populicola* assay, since 500 conidia μl^{-1} was the highest concentration studied for the two species. Comparison of the models for the two conidial treatments showed that species-specific PCR assays for *S. musiva* and *S. populicola* were more sensitive when used on DNA extracted from pure conidia from one species than on DNA extracted from a mixture of conidia of one target species with 500 conidia μl^{-1} from the other species. Obtaining a detection probability higher than 90% required a mean of 453 conidia μl^{-1} of *S. musiva* mixed with 500 conidia μl^{-1} of *S. populicola*, and 387 conidia μl^{-1} of *S. populicola* mixed with 500 conidia μl^{-1} of *S. musiva* (Table 2).

DISCUSSION

The PCR procedures described herein provide a new and a powerful tool for detecting, identifying and assessing the distribution of *Septoria musiva*, *S. populicola* and *S. populi*. The ability of the optimized PCR assays to differentiate between *S. musiva*, *S. populicola* and *S. populi* is attributable to the sequence divergence that occurs within the ITS region of these fungi. Since few point mutations distinguish the ITS sequences of *S. musiva* and *S. populicola*, the choice of primer sites for differentiating the two species was restricted. After stringency of the initial PCR conditions was increased, the two sets of PCR primers designed from four point mutations allowed the species-specific amplification of all *S. musiva* and *S. populicola* isolates tested. Sequencing of *S. populicola* isolates 02-86A, 02-79A and p02.01b and downloading of the sequences AY152583 to AY152587 from the NCBI databases revealed the occurrence of a nucleotide polymorphism located eight bases before the 3′ end of the forward primer specific to *S. populicola*. Perfect base-pairing between the 3′ end of the primer and the template is optimal for obtaining good results; minimal mismatch should exist within the last 5 to 6 nucleotides at the 3′ end of the primer (Dieffenbach, Lowe & Dveksler 1993). Although high specificity of the primer to the target sequence decreases mispriming (Vanichanon *et al*. 2000), experiments with sets of primers tested on defined DNA templates have shown that primers with a length between 17–20 nucleotides need at least three homologous nucleotides at their 3′ end for successful priming (Sommer & Tautz 1989, Adah *et al*. 1997). Our results show that presence of a single substitution eight bases before the 3′ end of the primer did not result in

Table 2. Parameters of the logistic regression model fitted to the percentage of positive results for each replicate set of experiments and number of spores required in the PCR assay for a 90% probability of detection estimated by the logistic regression model.

Serial dilution (primer set)	No. of PCR experiments	Value		Estimated no. of spores (mean and 95% confidence limits)		
		α	β	Mean	Lower	Upper
S. musiva (Smusf/r)	18	−0.8665 (±0.3522)	0.0185 (±0.0049)	166	84	427
S. populicola (Spopf/r)	16	−0.8144 (±0.3813)	0.0193 (±0.0056)	156	74	453
S. musiva (Smusf/r) with 500 *S. populicola* conidia/µl	14	−2.1162 (±0.4013)	0.0095 (±0.0023)	453	252	1000
S. populicola (Spopf/r) with 500 *S. musiva* conidia/µl	20	−0.6285 (±0.2724)	0.0073 (±0.0019)	387	206	960

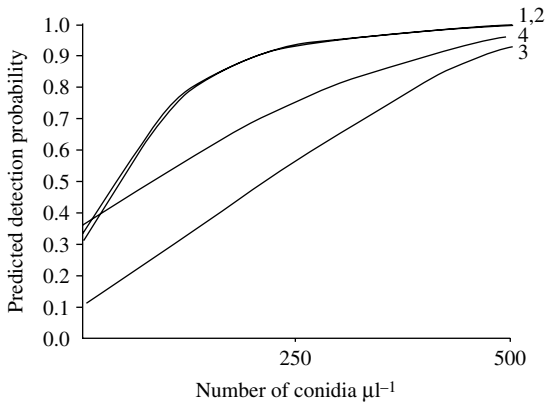

Fig. 4. Detection probability of *Septoria musiva* or *S. populicola* conidia using a single-step PCR with the species-specific primer pairs Smusf/r and Spopf/r. The probability of PCR detection for combination of primer pairs and conidial treatment was described by four logistic regression models, estimated from 68 observations using the maximum likelihood method. The logistic regression models estimated are illustrated by the solid lines for the primer pair Smusf/r on pure dilution of *S. musiva* conidia (1), for the primer pair Spopf/r used on pure dilution of *S. populicola* conidia (2), for the primer pair Smusf/r used on dilutions of *S. musiva* conidia in 500 conidia μl^{-1} of *S. populicola* (3), and for the primer pair Spopf/r used on dilutions of *S. populicola* conidia in 500 conidia μl^{-1} of *S. musiva* (4).

failure of amplification of the isolates sharing these point mutations. For *S. populi*, the occurrence of one gap and five nucleotide substitutions, not found in *S. musiva* and *S. populicola*, allowed us to design specific primers for this species. This level of variation between *S. populi* and the two other species of *Septoria* attacking poplar allowed for a lower stringency of the PCR conditions for *S. populi*-specific amplification. This primer pair was successfully tested on a limited collection of *S. populi* isolates sampled on *P. nigra* in Europe. The reduced size of this collection was due to the difficulty to obtain *S. populi* samples from other hosts and geographical locations or from international collections.

As the primary factors in developing a PCR detection protocol are specificity and sensitivity, it is essential that both the parameters used in the PCR reaction result in an optimal outcome (Innis & Gelfand 1990, Liew, Maclean & Irwin 1998). Due to the low number of differences between the primer pairs for *S. musiva* and *S. populicola*, cross-reactions were observed at low annealing temperatures (respectively under 63 ° and 60 ° with Université Laval protocol and 66 ° and 59 ° with University of Wisconsin-Madison protocol). Annealing temperatures, as well as concentrations of primers were thus optimized prior to verifying the specificity and determining the sensitivity of the PCR amplification. In testing for specificity, this current approach of testing known phylogenetically related species is justified primarily by the use of the nuclear rDNA. Designing primers from rDNA genes is far more reliable than the use of random non-defined probes or primers (Liew *et al.* 1998). The robustness of the particular primer pairs described herein was further demonstrated by the amplification from different isolates of *S. musiva* and *S. populicola* collected across North America. Similar results were obtained in all case where isolates were tested in both the Laval and Wisconsin-Madison laboratories. Furthermore, the specificity of these primers used under stringent conditions was verified by the absence of cross-reactivity with DNA from other *Septoria* species and other fungi from poplar tissues that might interfere with isolation of *S. musiva* (Stanosz & Stanosz 2002).

The design of specific primers from multicopy genes, such as the nuclear rDNA genes, adds greatly to the sensitivity of specific PCR detection (White *et al.* 1990, Bindslev, Oliver & Johansen 2002, Freeman *et al.* 2002). PCR assays that amplify rDNA should be more sensitive than those directed towards single-copy genes (Bindslev *et al.* 2002, Freeman *et al.* 2002). In order to confirm the sensitivity of the specific primer pairs we had designed from the ITS region of rDNA genes, we tested *S. musiva* and *S. populicola* total genomic DNA mixed in different ratios. Our specific primers successfully amplified target DNA even when the concentration of the latter in the mixture was 100-fold lower than the concentration of the competing DNA.

Direct PCR-based detection from fresh conidia is of particular interest because it avoids the time-consuming step of pathogen isolation and culture prior to the analysis; neither purification nor cultivation of the pathogen are required and specificity of tests is preserved. We tested three treatments in which conidia

of *S. musiva* and *S. populicola* were mixed in different ratios. Addition of untreated conidia of *S. musiva* or *S. populicola* directly to the PCR mixture was not a suitable option for detection of these pathogens. Detection was possible but unreliable because the sensitivity and the accuracy were poor, presumably due to the presence of inhibitors or because an insufficient amount of DNA was released from the conidia. Although heating may be sufficient for lysis of some types of spores, such as the thin-walled conidia of *Leptosphaeria maculans* (Williams, Ward & McCartney 2001), microscopic examination showed that conidia of *S. musiva* and *S. populicola* remained visibly intact after the initial 2 min denaturation step at 94 ° included in the PCR protocol used. Likewise, experiments in which untreated spores of *Penicillium roqueforti* were added directly to the PCR mixture showed that detection was possible but that sensitivity was poor because too few spores were disrupted by the initial 10-minutes step at 95 ° (Williams *et al.* 2001). Addition of mechanically disrupted conidia of *S. musiva* or *S. populicola* directly to the PCR mixture did not increase sensitivity and accuracy of PCR-detection of these pathogens. Although more than 50 % of the conidia of *S. musiva* and *S. populicola* were disrupted by using glass beads and heating, the PCR reactions appeared to be inhibited. Large amounts of PCR inhibitors present in the DNA samples can manifest themselves as complete reaction failure or as reduced sensitivity of detection (Wilson 1997, Kuske *et al.* 1998, Williams *et al.* 2001). Indeed, two elementary aspects of DNA amplification failure and generation of false negative results are the inability to expose the nucleic acids as target for amplification and inhibition of polymerase activity (Wilson 1997, Poussier *et al.* 2002, Valentine-Thon 2002). In the first case, loss of cell wall integrity may not be enough to permit amplification of DNA. Cell debris, proteins, and polysaccharides which result from lysis may cause inhibition by physical effects such as making the target DNA unavailable to the polymerase. In the second case, phenolic compounds from the sample, proteinases and denaturants used for cell lysis can inhibit the reaction by binding or denaturing the polymerase (Wilson 1997). Experiments with disrupted microbial cells and spores suggest that DNA purification is often necessary for suppressing inhibitors from the sample. Thus, incorporating DNA extraction and purification steps was reported to result in reliable detection of 250 ml^{-1} (about 25 spores in the PCR mixture) spores of *Bacillus globigii* using 35 PCR cycles (Kuske *et al.* 1998), 100 spores of *P. roqueforti* using nested PCR (Williams *et al.* 2001) and DNA equivalent of 10 ascospores of *S. sclerotiorum* added to PCR using a single-step amplification protocol (Freeman *et al.* 2002). The combination of extraction and purification of DNA was also found to be the best method to produce a template for PCR amplification from *S. musiva* or *S. populicola* conidial suspensions. Using the combination of the DNA extraction using hot-detergent treatment, beads mill homogenization and the DNA purification protocol modified from Lee & Taylor (1990), it was possible to detect accurately with a probability of 90 % less than 170 conidia of *S. musiva or S. populicola* with the single-step PCR protocol developed in the Université Laval laboratory. Non target DNA may also be a cause of failed PCR reaction by sequestration of primers (Wilson 1997). Comparison of the logistic linear models estimated for the different combinations of primer pairs and conidial treatment showed that the sensitivity was improved if we used DNA extracted from pure conidia rather than DNA extracted from conidia of the target species mixed with conidia from the other species. Reduction of sensitivity may be explained by the occurrence of large amounts of non-target DNA extracted from the non-target species. Indeed, we observed (Fig. 3) that the amplification sensitivity for DNA extracted from mycelia of the target species mixed with DNA from the other species was 10 fold lower than for pure DNA from the target species.

When considering the sensitivity of the specific primer pairs using DNA extracted and purified directly from conidia, it was important to differentiate the number of conidia used in the extraction and the number actually used in the PCR detection assay. For example, it was possible to detect DNA with accuracy using the equivalent of 100 spores of *P. roqueforti* by nested PCR, but, in order to achieve this, the large scale DNA extraction used required 4000 spores to be processed (Williams *et al.* 2001). The detection of DNA equivalent of 10 ascospores of *S. sclerotiorum* added to PCR using a single-step amplification protocol required processing of 400 spores for the DNA extraction and purification protocol (Freeman *et al.* 2002). In the case of *S. musiva* and *S. populicola*, rather than extracting DNA from a large number of spores and then diluting it in order to assess detection efficiency, serial dilutions of conidia were used in the DNA extraction protocol. Moreover, the accurate detection of these two species required processing of 34 000 conidia in 200 µl of solution during the extraction and purification preparation. The counting of *S. musiva* conidia from leaf spots on *P. deltoides* and *P.* ×*jackii* sampled in Sainte-Foy in October, at the end of the epidemic season, revealed that leaf spots with one to nine pycnidia (mean 4.23 pycnidia +/− 2.38) contained 1500–36 000 conidia (mean 8813 conidia +/− 8473) with a median value of 6400 conidia. Thus, it appears that the PCR assay for *S. musiva* can potentially detect DNA extracted from *S. musiva* conidia when using the DNA extraction and purification protocols with conidia collected on a mean of 4 leaf spots. However, since experiments were conducted on conidia from fungi cultured *in vitro*, further experiments on environmental samples are required in order to determine definitively the ability of the primer sets to amplify DNA of conidia from leaf spots or canker.

The specific primers developed in this study allow reliable and unambiguous identification of *S. musiva*,

S. populicola and *S. populi*. Two PCR protocols were developed with success at two different institutions, thereby showing that the results obtained were robust with respect to minor differences in laboratory procedures. Moreover, competitive assays carried out on mixtures of *S. musiva* and *S. populicola* DNA confirmed that the molecular approach was sensitive relatively to the PCR technique used (single PCR i.e. non-nested or non-quantitative PCR methods). Thus, our PCR-assay provides an accurate and easily transferable method for the identification of these poplar fungi. One of the first practical applications of the PCR-primers described here has been in the confirmation of isolate identities used in population genetic studies (Feau *et al*., 2005). In addition, the sensitivity of the primers allows ability for rapid detection of both *S. musiva* and *S. populicola* in cases where both species are present on the same hybrid poplar tissues. Moreover, the use of this PCR assay directly on DNA extracted and purified from conidia would be extremely useful to detect, predict and monitor the presence and spread of these pathogens, both on wild poplars or on hybrids in plantations. Accurate information on host range and distribution of *Septoria* species would, in turn, provide a valuable tool for breeders and phytopathologists, and help them better understand the epidemiology of these pathogens and the etiology of the diseases they cause.

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